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An Optimized CD4 T-Cell Response Can Control Productive and Latent Gammaherpesvirus Infection

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CD4 T cells are important for control of infection with murine gammaherpesvirus 68 (γHV68), but it is not known whether CD4 T cells function via provision of help to other lymphocyte subsets, such as B cells and CD8 T cells, or have an independent antiviral function. Moreover, under conditions of natural infection, the CD4 T-cell response is not sufficient to eliminate infection. To determine the functional capacities of CD4 T cells under optimal or near-optimal conditions and to determine whether CD4 T cells can control γHV68 infection in the absence of CD8 T cells or B cells, we studied the effect of ovalbumin (OVA)-specific CD4 T cells on infection with a recombinant γHV68 that expresses OVA. OVA-specific CD4 T cells limited acute γHV68 replication and prolonged the life of infected T-cell receptor-transgenic RAG (DO.11.10/RAG) mice, demonstrating CD4 T-cell antiviral activity, independent of CD8 T cells and B cells. Despite CD4 T-cell-mediated control of acute infection, latent infection was established in DO.11.10/RAG mice. However, OVA-specific CD4 T cells reduced the frequency of latently infected cells both early (16 days postinfection) and late (42 days postinfection) after infection of mice containing CD8 T cells and B cells (DO.11.10 mice). These results show that OVA-specific CD4 T cells have B-cell and CD8 T-cell-independent antiviral functions in the control of acute infection and can, in the absence of preexisting CD8 T-cell or B-cell immunity, inhibit the establishment of gammaherpesvirus latency.

After the acute infection is cleared, gammaherpesviruses establish a chronic equilibrium with the host such that the immune system prevents the virus from overwhelming the host but fails to eliminate the infection. Viral contributions to this equilibrium include genes that provide to the capacity to establish, maintain, and reactivate from latent infection. Host contributions to the this equilibrium include the capacity of CD4 T, CD8 T, and B cells to control acute and latent virus infection. Disruption of this equilibrium has serious consequences during chronic infection, including development of virus-associated tumors and vascular disease in different hosts (40, 49, 51, 55).

To better understand gammaherpesvirus pathogenesis and immunity and to identify the immune mechanisms that contribute to the maintenance of the host-virus equilibrium during chronic infection, we and others have studied murine gammaherpesvirus 68 (γHV68). γHV68 infects inbred and outbred strains of mice and establishes latency, and chronic γHV68 infection is associated with induction of tumors and with severe arteritis in immunodeficient mice (40, 51; F. Suarez, M. Jacoby, S. A. Tibbetts, S. H. Speck, and H. W. Virgin IV, unpublished results), providing a good system to dissect the immune and viral requirements for chronic infection and disease.

During acute infection, γHV68 replication is detectable in multiple organs by plaque assay. By 16 days postinfection, acute γHV68 infection is contained and a latent infection of B cells, macrophages, and dendritic cells is established (13, 39, 50, 52, 53, 54). Latent infection is characterized by the presence of viral genome-positive cells and the absence of high levels of lytic virus (14, 26, 53). In certain immune-deficient mice, a higher level of infectious virus (persistent replication) is produced during chronic infection (18, 43, 48).

CD4 T cells are essential for maintaining the equilibrium between virus and host during acute and chronic infection (7). Depletion of CD4 T cells in addition to CD8 T cells worsens acute γHV68 infection (12). In addition, CD4 T cells are critical for control of chronic infection. Mice that lack CD4 T cells, due to a lack of major histocompatibility complex (MHC) class II, initially clear the infection but then exhibit increasing persistent infection over weeks to months, eventually leading to severe arteritis and death of the animal (6, 7, 51). The epitopes that these antiviral CD4 T cells recognize are not well-defined. Two viral epitopes, $gp150_{67\text{-}83}$ and ORF $11_{168\text{-}180}$, which account for 10 to 20% of the total γHV68-specific CD4 T-cell response, have been identified in C57BL/6 mice (16).

While there is evidence that CD4 T cells play an important role during acute and chronic infection, the mechanisms responsible for CD4 T-cell action during acute and chronic infection are not well understood. As both CD8 T cells and B cells are important for limiting the frequency of latently infected cells (17, 19, 43, 50) and responses of these cells either depend on or are regulated by CD4 T cells, it is possible that CD4 T cells function by regulating the antiviral activity of other cells. CD4 T cells are necessary for the expansion of virus-specific and nonspecific T and B cells that occurs during

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γHV68 infection (6, 15, 20, 38) and are necessary for activation of B cells during infection (38). Furthermore, CD4 T cells may be the source of CD40L expression required to maintain latency in memory B cells (14, 20, 54).

A second, but not mutually exclusive, possibility is that CD4 T cells act independently from CD8 T cells and B cells to control γHV68 infection. Experiments by Christensen et al. suggested that CD4 T cells have such an independent role in controlling infection (8). By depleting CD4 and CD8 T cells in B cell$^{-/-}$ mice, they showed that CD4 T cells contribute to the control of replication in the lung in a gamma interferon (IFN-γ)-dependent manner.

To better understand how CD4 T cells contribute to control of acute and latent γHV68 infection, we developed a model system in which a heterologous protein, ovalbumin (OVA), was expressed from the v-cyclin locus. The form of OVA that we used in these experiments is efficiently presented to CD4 T and CD8 cells (2, 21). We then used this virus to infect T-cell receptor (TCR) transgenic (tg) mice that had CD4 T cells specific for OVA. We reasoned that we could use this system to dissect, under optimal conditions of high levels of antigen expression and high T-cell frequency, the potential roles of CD4 T cells in the control of γHV68 infection. Using this system, we determined whether the antiviral actions of CD4 T cells require B cells and CD8 T cells. We found that CD4 T cells, in the absence of CD8 T cells and B cells, are sufficient to prolong the lives of infected mice and to reduce viral titers in multiple organs. While CD4 T cells were sufficient to reduce viral titers, they were not sufficient in the absence of CD8 T cells and B cells to prevent a latent infection from being established. However, in conjunction with nonimmune CD8 T and B cells, high levels of immune CD4 T cells, present at the time of infection, can decrease the establishment of both early and late forms of γHV68 latency. These data, in concert with results obtained by McClellan et al. (26a), show an important role for CD4 T cells in control of chronic γHV68 infection.

MATERIALS AND METHODS

Mice. Mice were housed and bred under specific-pathogen-free conditions at the Washington University School of Medicine in accordance with all federal and university guidelines. C57BL/6 (B6), BALB/c, and DO.11.10 (BALB/c background [29]) mice were obtained from Jackson Labs. OTII mice (B6 background [1]) were a generous gift from Paul Allen (Washington University). Rag1$^{-/-}$ mice (B6 background [27]) and Rag2$^{-/-}$ mice (Taconic Farms) (BALB/c background [34]) were maintained in our mouse colony. DO.11.10/RAG and OTII/RAG mice were bred in our facility by crossing DO.11.10 or OTII mice with Rag2$^{-/-}$ or Rag1$^{-/-}$ mice and screening progeny by flow cytometry. For all experiments, mice were at least 8 weeks of age and were age and sex matched within experiments.

Viruses and viral assays. γHV68.v-cyclin.LacZ (47) and γHV68.v-cyclin.OVA (D. C. Braaten, R. Sparks-Thissen, S. Kreher, S. H. Speck, and H. W. Virgin IV, unpublished data) (referred to here as γHV68.LacZ and γHV68.OVA, respectively) were passaged in NIH 3T12 fibroblasts (50). Mice were infected with 100 PFU of virus by intraperitoneal infection (41). Tissues were harvested, and viral titers were determined by plaque assay (50). Briefly, organs were frozen in 1 ml of medium, thawed, and then homogenized together with 100 μl of 1.0-mm-diameter zirconia-silica beads (Biospec, Inc.) at 3,200 rpm for 2 min with a Mini-Bead-Beater-8 (Biospec Inc.) prior to plaque assay. All titers were determined in parallel with a viral stock of known titer. The limit of detection of the assay used is 50 PFU per organ.

Quantitation of latency and persistent replication. The frequency of cells reactivating from latency and the level of persistent viral replication were determined as described previously (41, 50). Briefly, cells were pooled from four or five mice per group and then plated in serial twofold dilutions (24 wells per dilution) starting at 1×10^5 cells/well for splenocytes and 4×10^4 cells/well for peritoneal cells onto an indicator monolayer of mouse embryo fibroblasts in 96-well plates. Reactivation was detected as cytopathic effect in the mouse embryo fibroblast monolayers over 21 days of culture. To detect preformed infectious virus (persistent replication) parallel samples were mechanically disrupted prior to plating. This procedure kills >99% of the cells but has at most a twofold effect on viral titer (52), allowing for distinction between reactivation from latency (which requires live cells) and persistent viral replication. To determine the frequency of cells carrying the viral genome, single-copy sensitivity nested-PCR assays for γHV68 gene 50 were performed on serial dilutions of cells as previously described (43). Briefly, cells were serially diluted threefold starting at 10^4 cells per reaction in a background of uninfected 3T12 cells such that a total of 10^4 cells were present in each sample. Single copies of a plasmid containing gene 50 in a background of 3T12 cells were included as a positive control. 3T12 samples with no plasmid were included as a negative control. After overnight lysis of cells in proteinase K, nested PCR was performed and products were visualized on a 1.5% agarose gel. For the experiments reported here, all assays demonstrated approximately single-copy sensitivity, with no false positives (frequencies for control plasmids were as follows: 10 copies, 348 of 366 positive [95%]; 1 copy, 216 of 366 [60%]; 0.1 copy, 28 of 366 [7%]).

Flow cytometry. Flow cytometry was performed on a Becton Dickinson FACSCalibur, and 200,000 events were collected per sample as described previously (46). The following monoclonal antibodies were used: fluorescein isothiocyanate–anti-Vα2 (BD Biosciences catalog no. 553288), fluorescein isothiocyanate–anti-DO.11.10 TCR (KJ1-26; Caltag Laboratories catalog no. MM7501), phycoerythrin–anti-CD44 (BD Biosciences catalog no. 553134), phycoerythrin–anti-CD62L (Caltag Laboratories catalog no. RM4304), and Cy-chrome–anti-CD4 (BD Biosciences catalog no. 553654). For analysis of activation markers, cells were first gated on live cells, using forward scatter and side scatter, and then gated on KJ1-26$^+$ CD4$^+$ cells.

Depletion of T cells in vivo. YTS 191 (anti-CD4) (9), SFR3-DR5 (anti-HLA-DR5, isotype control) (32), and H35 (anti-CD8) (35) were grown in HyQ ADCF-Ab medium (HyClone) in CL1000 flasks (Integra Biosciences, Ltd.). Five hundred micrograms of antibody per mouse was injected intraperitoneally every 4 to 5 days. Depletions were greater than 90% effective as determined by flow cytometry (data not shown).

Statistical methods. All data were analyzed with Prism software (GraphPad Software, San Diego, Calif.). Frequencies of reactivating and genome-positive cells were calculated on the basis of the Poisson distribution by determining the cell number at which 63.2% of the wells scored positive. The frequency of cells containing preformed virus was calculated as previously described (43). The frequencies of reactivating cells and genome-positive cells were compared by use of the paired *t* test. Mice were scored as dead at the time of death or when sacrificed if moribund, and survival data were analyzed by use of the Mantel-Haenzsel test, with death as the primary variable. Acute titer data were analyzed by using the Mann-Whitney test. All error bars in the figures represent the standard errors of the means.

RESULTS

We sought to determine whether virus-specific CD4 T cells, under optimized conditions, can limit lethal infection, acute replication, persistent replication, and establishment of γHV68 latency by using a combination of γHV68.OVA and TCR tg mice whose CD4 T cells are specific for an OVA peptide. OVA was chosen as the model antigen for these studies due to the availability of two strains of TCR tg mice whose CD4 T cells recognize an OVA peptide in the context of MHC class II. DO.11.10 TCR tg mice are on the BALB/c background and contain tg CD4 T cells that recognize amino acids 323 to 339 of OVA together with MHC class II I-A^d (29, 33). OTII TCR tg mice are on the B6 background and contain tg CD4 T cells that recognize the same epitope together with MHC class II I-A^b (1, 33). To determine whether antiviral activities of CD4 TCR tg T cells in this optimized system require CD8 T cells or B cells, we bred the DO.11.10 and the OTII TCR transgenes onto the T- and B-cell deficient RAG background, generating

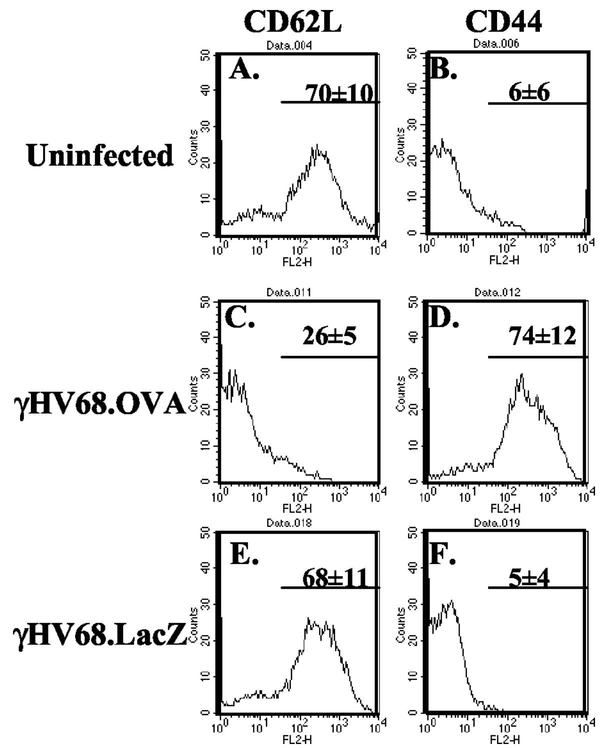

FIG. 1. DO.11.10 T cells are activated during infection with γHV68.OVA. DO.11.10/RAG mice were infected for 16 days with either γHV68.OVA or γHV68.LacZ. Splenocytes were isolated and stained with antibodies against CD4, the DO.11.10 TCR (KJ1-26), and either CD62L or CD44. Cells were gated on KJ1-26$^+$ CD4$^+$ cells prior to analysis (approximately 10% of the total splenocytes). Numbers in the graphs represent the percentage of cells staining positive ± standard error of the mean from four independent experiments.

mice expressing only CD4 T cells specific for OVA (data not shown).

γHV68.OVA-infected cells are recognized by TCR tg CD4 T cells. γHV68.OVA expresses a fusion protein of OVA with the transferrin receptor transmembrane domain, which is efficiently processed and presented to both CD4 and CD8 T cells (21, 22). Expression of this fusion protein is driven at a high level by the cytomegalovirus immediate-early promoter. The expression cassette was inserted into the v-cyclin locus. γHV68.OVA makes OVA during in vitro and in vivo infection, grows normally in vitro, and is recognized by OTI TCR tg CD8 T cells specific for OVA (Braaten et al., unpublished data). γHV68.LacZ expresses the *lacZ* gene from the same locus and was used as a control for antigen specificity (47).

To determine whether OVA expressed in γHV68.OVA-infected cells was processed and presented on I-A^d, DO.11.10/RAG mice were left uninfected or were infected with γHV68.OVA or γHV68.LacZ. Sixteen days later, splenic CD4 T cells were stained for activation markers (CD44 and CD62L). DO.11.10 T cells from uninfected or γHV68.LacZ-infected mice were not activated, as shown by high levels of CD62L and low levels of CD44 (Fig. 1A, B, E, and F). In contrast, DO.11.10 T cells from γHV68.OVA-infected mice were activated, as shown by high levels of CD44 (Fig. 1D) and low levels of CD62L (Fig. 1C). This demonstrated that DO.11.10 T cells are activated in an antigen-specific manner during infection with γHV68.OVA.

It was of interest to compare the frequencies of virus-specific CD4 T cells in this optimized system to the CD4 T-cell response during γHV68 infection. During infection, approximately 1 of 48 CD4 T cells are specific for γHV68 antigens at 21 days postinfection (16). Based on published estimates of the number of virus-specific CD4 T cells (50), we estimate that there are approximately 4.5×10^5 γHV68-specific CD4 T cells at 21 days postinfection. In contrast, approximately 2×10^6 CD4 T cells in the DO.11.10/RAG mice (data not shown) and 1×10^7 CD4 T cells in the DO.11.10 (11) mice are OVA specific. This suggests that, in our model system, there are approximately 5-fold more γHV68.OVA-specific CD4 T cells in DO.11.10/RAG mice and approximately 22-fold more γHV68.OVA-specific CD4 T cells than the number of γHV68-specific CD4 T cells found during γHV68 infection.

OVA-specific CD4 T cells inhibit lethal γHV68 infection in the absence of CD8 T cells or B cells. We next determined whether OVA-specific CD4 T cells were sufficient to protect against lethal infection by infecting DO.11.10/RAG mice with γHV68.OVA. DO.11.10/RAG mice infected with γHV68.OVA survived an average of 10 more days than DO.11.10/RAG mice infected with the control virus, γHV68.LacZ (mean time to death, 29 days; $P < 0.0001$) (Fig. 2A). This effect was not due to attenuation of γHV68.OVA, as γHV68.OVA and γHV68.LacZ were equally virulent in BALB/c RAG mice (Fig. 2B) ($P = 0.7712$). The effect of the DO.11.10 CD4 T cells was OVA specific, since γHV68.LacZ was equally virulent in both BALB/c RAG and DO.11.10/RAG mice (Fig. 2A and B) ($P = 0.7712$). Together, these data demonstrate that CD4 T cells are sufficient to prolong the life span of the mouse after γHV68 infection in the absence of detectable CD8 T cells and B cells.

To confirm these results for a different mouse background and MHC haplotype, we also performed experiments with OTII/RAG mice. OTII/RAG mice infected with γHV68.OVA survived an average of 10 days longer than mice infected with γHV68.LacZ ($P = 0.0031$) (Fig. 2C). B6 RAG mice infected with either γHV68.OVA or γHV68.LacZ survived approximately 20 days, with no significant difference in survival between the two viruses ($P = 0.1518$), confirming that the OVA virus was not attenuated in B6 RAG mice (Fig. 2D). Similarly, B6 RAG mice and OTII/RAG mice infected with γHV68.LacZ had a mean time to death of 20 days ($P = 0.4880$), demonstrating that the effect of OTII CD4 T cells is OVA specific. (Fig. 2C and D). These experiments confirmed that OVA-specific CD4 T cells mediated increased survival following γHV68 infection in the absence of detectable CD8 T cells and B cells.

To confirm that the increased survival of DO.11.10/RAG mice after infection was due to the presence of CD4 T cells, we depleted CD4 T cells and then infected the mice with γHV68.OVA. As controls, mice were left untreated or were treated with an isotype control antibody or antibody specific for CD8. Untreated mice and mice treated with either the isotype control or anti-CD8 antibodies died with approximately the same kinetics (Fig. 3). However, mice treated with anti-CD4 died an average of 10 days faster than untreated ($P < 0.0001$), isotype control-treated ($P = 0.0012$), or anti-CD8-treated mice ($P = 0.0001$) (Fig. 3). Together, these data demonstrated that CD4 T cells can delay lethal

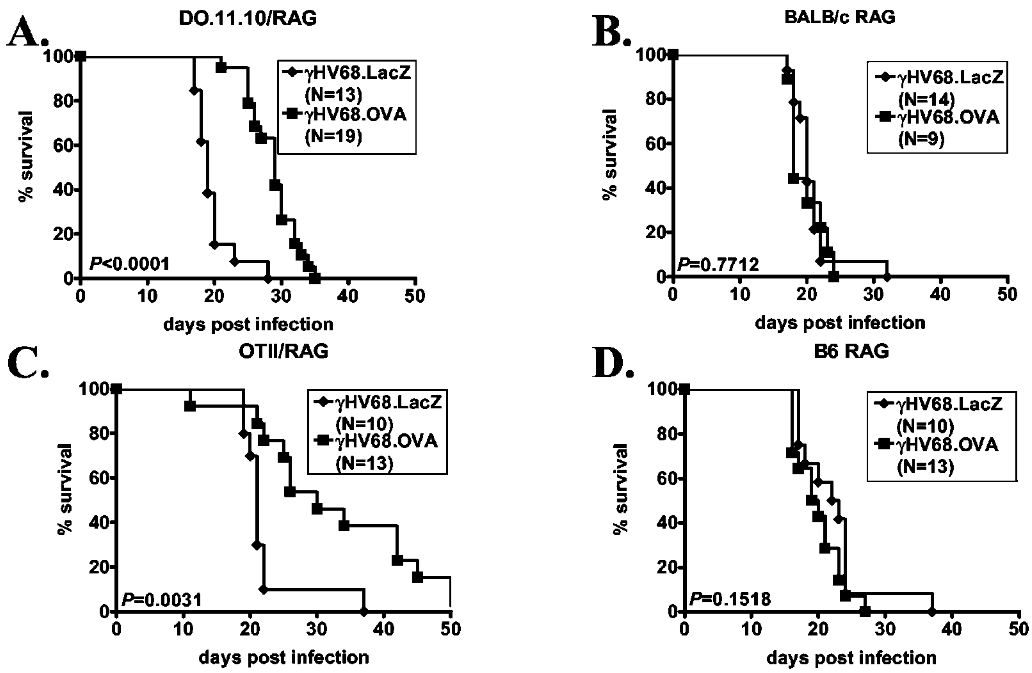

FIG. 2. OVA-specific CD4 T cells partially protect against lethality in RAG mice. DO.110/RAG (A), BALB/c RAG (B), OTII/RAG (C), or B6 RAG (D) mice were infected with either γHV68.OVA or γHV68.LacZ and monitored for survival from infection. The data shown here were pooled from three independent experiments. N, number of mice per group.

γHV68 infection in the absence of CD8 T cells or B cells. However, CD4 T cells were not sufficient to completely protect mice from γHV68 infection.

OVA-specific CD4 T cells eliminate detectable replication of γHV68 at 16 days after infection. To determine whether the increased life spans of infected DO.11.10/RAG mice correlated with reduced viral replication in these mice, we measured the amount of virus in the spleen and liver at 16 days after infection with either γHV68.OVA or the control virus, γHV68.LacZ. Both viruses grew to equivalent titers in the spleens ($P = 0.9372$) and livers ($P = 0.7618$) of RAG mice (Fig. 4B and D). In addition, γHV68.LacZ grew to equivalent titers in the spleens ($P = 0.6282$) and livers ($P = 0.1797$) of both DO.11.10/RAG and RAG mice, demonstrating that the OVA-specific CD4 T cells did not alter the titer of a virus that does not express the OVA epitope (Fig. 4A and C). In contrast, DO.11.10/RAG mice infected with γHV68.OVA had 1,000-fold-lower titers of virus in the liver ($P = 0.0002$) and 100-fold-lower titers in the spleen ($P = 0.0001$) than RAG mice (Fig. 4A and C). Viral titers in DO.11.10/RAG mice infected with γHV68.OVA were at or below the level of sen-

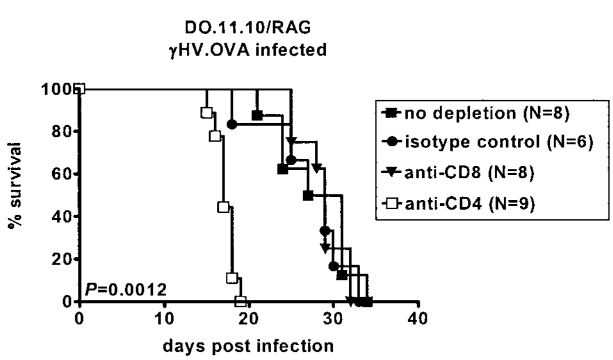

FIG. 3. Survival from infection in DO.11.10/RAG mice is dependent on CD4 T cells. DO.11.10/RAG mice were left untreated or treated with isotype control, anti-CD8, or anti-CD4 and then infected with γHV68.OVA and assayed for survival from infection. Data were obtained from two independent infections. N, number of mice per group.

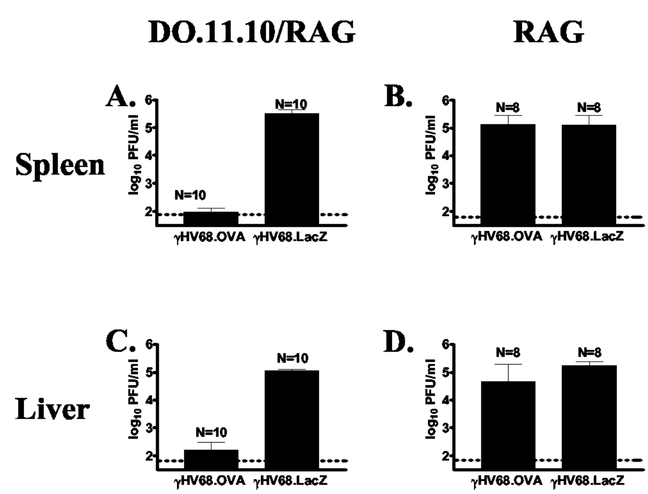

FIG. 4. OVA-specific CD4 T cells control virus titer at 16 days postinfection. DO.11.10/RAG (A and C) or RAG (B and D) mice were infected with either γHV68.OVA or γHV68.LacZ. At 16 days postinfection, spleens (A and B) and livers (C and D) were harvested and the amount of virus was quantitated by plaque assay. N, number of mice per group. The dashed lines indicate the limit of sensitivity of the plaque assay. Data were pooled from two independent experiments.

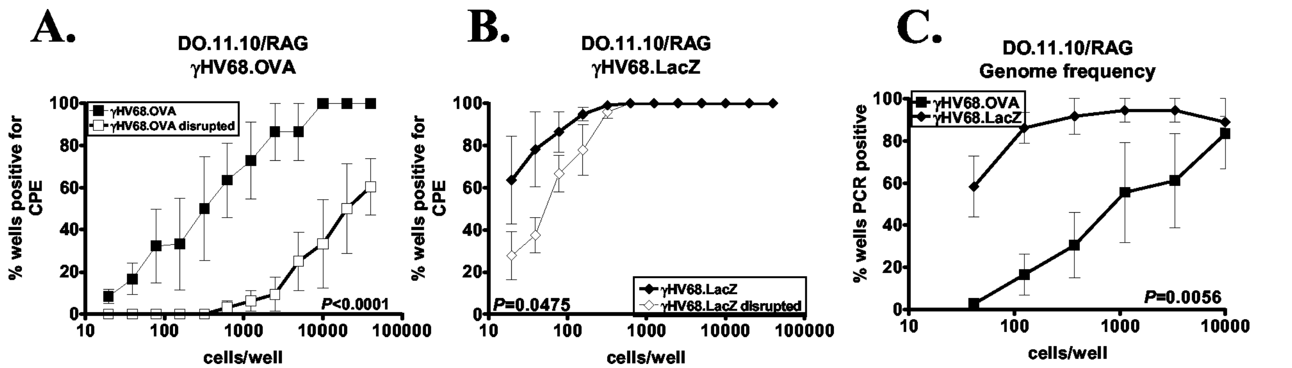

FIG. 5. A latent infection is established after infection with γHV68.OVA in DO.11.10/RAG mice. DO.11.1O/RAG mice were infected with either γHV68.OVA (A and C) or γHV68.LacZ (B and C). At 16 days after infection, peritoneal cells were harvested and subjected to limiting-dilution ex vivo reactivation (A and B) or limiting-dilution PCR (C) assays. Data were pooled from four independent experiments.

sitivity of the plaque assay. Only 4 of 10 DO.11.10/RAG mice infected with γHV68.OVA had detectable virus in the spleen, and only 3 of 10 had detectable virus in the liver. These data demonstrated that OVA-specific CD4 T cells are sufficient to control γHV68.OVA replication, even in the absence of CD8 T cells or B cells.

Latent infection is established in mice containing only OVA-specific CD4 T cells. The experiments described above show that OVA-specific CD4 T cells efficiently controlled replication of γHV68.OVA in the absence of CD8 T cells and B cells (Fig. 4). However, it was possible that despite efficient control of viral replication, γHV68.OVA was still capable of establishing latency in DO.11.10/RAG mice. We therefore considered the hypothesis that CD4 T cells would be sufficient to establish, in the absence of B cells and CD8 T cells, an equilibrium between the virus and host in which replication was controlled but latency was present.

To test this hypothesis, we infected DO.11.10/RAG mice with γHV68.OVA or γHV68.LacZ and measured the quantity of preformed infectious virus and latent virus 16 days after infection. Since the presence of preformed virus would potentially obscure detection of latency, we first looked at the amount of preformed virus in both samples. Peritoneal cells from γHV68.OVA-infected DO.11.10/RAG mice contained 400-fold-less preformed virus than cells from γHV68.LacZ-infected mice (Fig. 5A and B) ($P < 0.0001$). This is consistent with the decrease in viral titers seen in spleen and liver 16 days after infection as measured by plaque assay (Fig. 4). The decrease in the amount of preformed γHV68.OVA in samples from DO.11.10/RAG mice was dependent on CD4 T cells, since depletion of CD4 T cells (Fig. 6C) (but not treatment with an isotype control or anti-CD8 antibody [Fig. 6A and B]) resulted in an increase in preformed virus to levels seen after infection with γHV68.LacZ ($P = 0.0001$). Thus, CD4 T cells control persistent infection in peritoneal cells in the absence of B cells or CD8 T cells.

We next evaluated the frequency of latently infected cells in DO.11.10/RAG mice infected with either γHV68.OVA or γHV68.LacZ. The level of preformed virus in peritoneal cells of γHV68.OVA-infected DO.11.10 mice was sufficiently low to allow us to detect a significant level of viral latency (Fig. 5A). Latency was also detected after depletion with either an isotype control or anti-CD8 antibody (Fig. 6A and B) but not after treatment with anti-CD4 (Fig. 6C), demonstrating that CD4 T cells were necessary for detection of latency. These data correlate with the demonstration that in DO.11.10/RAG mice there were 40-fold fewer viral genome-bearing peritoneal cells after infection with γHV68.OVA than after infection with γHV68.LacZ ($P = 0.0056$) (Fig. 5C). This indicates that a latent infection was established in the presence of OVA-specific CD4 T cells. However, these experiments did not allow us to compare the levels of γHV68.OVA latency in the presence

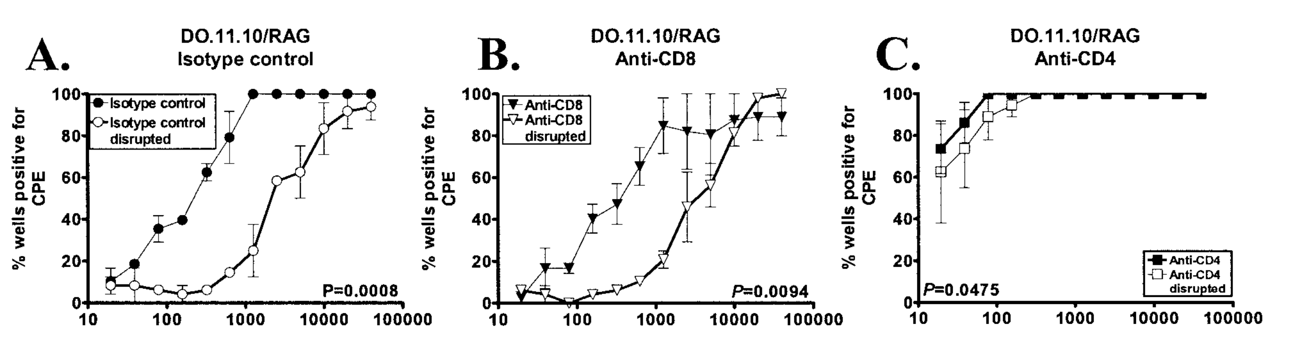

FIG. 6. Latent infection in DO.11.10/RAG mice is dependent on CD4 T cells. Prior to infection with γHV68.OVA, DO.11.10/RAG mice were treated with isotype control (A), anti-CD8 (B), or anti-CD4 (C) antibodies. At 16 days after infection, peritoneal cells were harvested and subjected to limiting-dilution ex vivo reactivation assays. Data were pooled from three independent experiments.

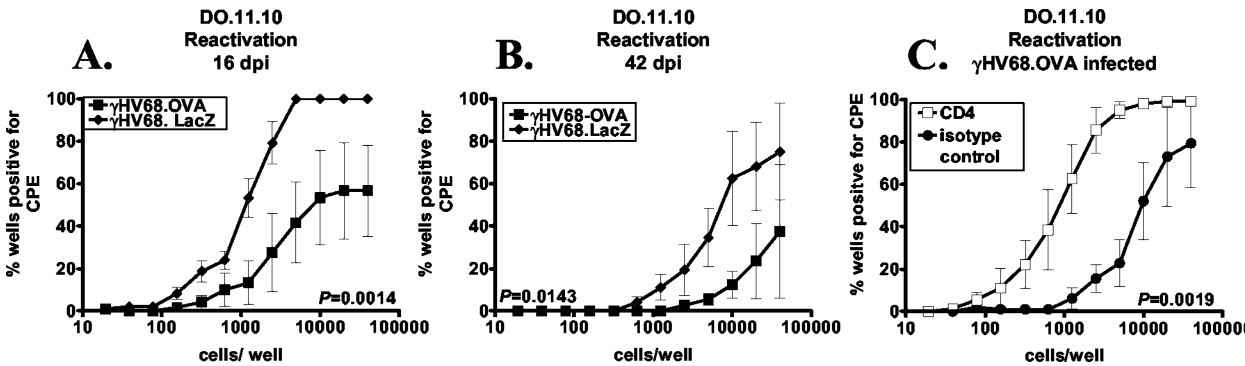

FIG. 7. OVA-specific CD4 T cells reduce the frequency of peritoneal cells reactivating ex vivo. DO.11.10 mice were infected with either γHV68.OVA or γHV68.LacZ for either 16 (A and C) or 42 (B) days. Peritoneal cells were harvested and subjected to limiting-dilution reactivation assays. For panel C, DO.11.10 mice were treated with anti-CD4 or isotype control antibodies prior to infection with γHV68.OVA. Data were pooled from three independent experiments.

and absence of OVA-specific T cells. This is because the high levels of γHV68.OVA replication in RAG mice and the high levels of γHV68.LacZ replication in DO.11.10/RAG mice precluded quantification of latency.

CD4 T cells reduce the frequency of cells that reactivate ex vivo from latency. It has been reported that epitope-specific CD8 T cells limit early γHV68 latency (assayed 16 days after infection) but not late γHV68 latency (assayed 42 days after infection) (4, 24, 37, 45). It was of interest to determine whether epitope-specific CD4 T cells could limit both early and late γHV68 latency. Since we were unable to determine whether OVA-specific CD4 T cells inhibited the establishment of γHV68.OVA latency in DO.11.10/RAG mice, we addressed this question by using DO.11.10 mice, which contain high levels of OVA-specific CD4 T cells prior to infection but also contain nonimmune CD8 T cells and B cells. We hypothesized that high numbers of preexisting CD4 T cells specific for OVA would inhibit the establishment of γHV68 latency in DO.11.10 mice.

To test this hypothesis, we infected DO.11.10 mice with γHV68.OVA or γHV68.LacZ and determined the effect of OVA-specific CD4 T cells on the frequency of cells that reactivate from latency ex vivo. At 16 days postinfection, the frequency of peritoneal cells reactivating from latency in DO.11.10 mice infected with γHV68.OVA was decreased 10-fold compared to that in γHV68.LacZ-infected mice (P = 0.0014) (Fig. 7A). DO.11.10 mice infected for 42 days with γHV68.OVA also showed a significant (P = 0.0143) decrease in the frequency of cells reactivating from latency compared to γHV68.LacZ-infected mice (Fig. 7B). This was not due to a defect in the capacity of γHV68.OVA to reactivate, as reactivation of the two viruses was equivalent in BALB/c mice (data not shown). No preformed virus was detected after infection with either virus in DO.11.10 mice (data not shown). These data suggested that OVA-specific CD4 T cells inhibit ex vivo reactivation from both early and late γHV68 latency.

To determine if this reduction in reactivation from latency was dependent on CD4 T cells, we depleted CD4 T cells from both γHV68.OVA- and γHV68.LacZ-infected mice and measured the frequency of peritoneal cells reactivating from latency. DO.11.10 mice infected with γHV68.LacZ had similar frequencies of cells reactivating from latency whether the mice were treated with an isotype control antibody or anti-CD4 (data not shown). Mice treated with anti-CD4 and then infected with γHV68.OVA had a 14-fold increase in the frequency of cells reactivating from latency compared to mice treated with the isotype control (Fig. 7C) (P = 0.0019), demonstrating that the decrease in the frequency of cells reactivating from latency is dependent on CD4 T cells. Together, these data demonstrated that CD4 T cells can effectively limit the frequency of cells that reactivate ex vivo from γHV68 latency.

CD4 T cells reduce the establishment of γHV68 latency. CD4 T-cell effects on the frequency of cells that reactivate from latency could be explained either by effects on the frequency of cells that carry the latent virus (establishment of latency) or by alterations in the efficiency with which latently infected cells reactivate in ex vivo assays (43). We therefore tested the hypothesis that OVA-specific CD4 T cells decrease the frequency of cells carrying the viral genome. The frequency of cells carrying the γHV68.OVA genome was decreased significantly at both 16 (30-fold; P = 0.0044) and 42 (P = 0.0001) days after infection for γHV68.OVA compared to γHV68.LacZ (Fig. 8A and B). This was not due to a defect in the capacity of γHV68.OVA to establish infection, as the frequencies of genome-positive cells after infection with both viruses were equivalent in BALB/c mice (data not shown). In addition, DO.11.10 mice pretreated with anti-CD4 and then infected with γHV68.OVA had a 12-fold increase in the frequency of cells containing the viral genome compared to mice pretreated with an isotype control antibody (P = 0.0045) (Fig. 8C). This frequency was similar to that for γHV68.LacZ-infected mice treated with either control antibody or anti-CD4 (data not shown). These results demonstrated that the presence of virus-specific CD4 T cells at the initiation of infection can limit the establishment of both the early and late latency but that even an optimized CD4 T-cell response to a single virus-encoded protein cannot completely prevent establishment of latency.

DISCUSSION

In this report, we demonstrate significant antiviral activities of CD4 T cells, in the absence of B cells or CD8 T cells, in the control of acute and chronic γHV68 infection. These CD4 T-cell helper function-independent antiviral effects were man-

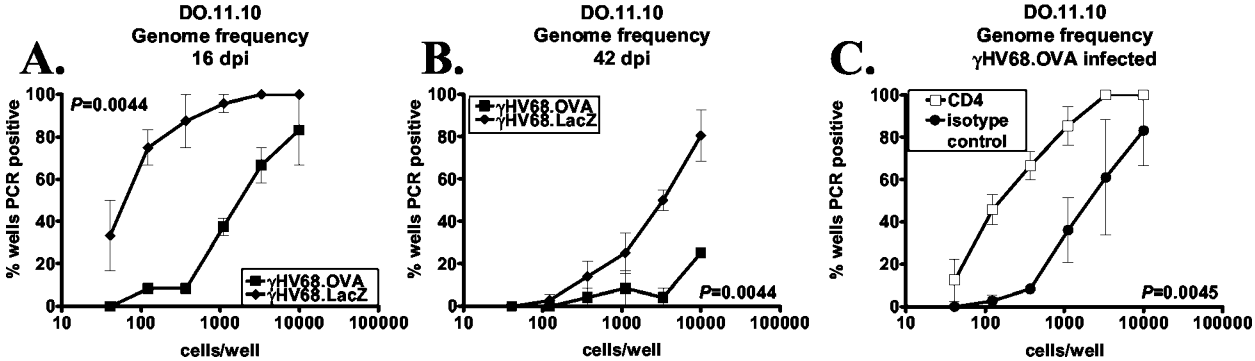

FIG. 8. OVA-specific CD4 T cells reduce the frequency of peritoneal cells carrying the viral genome. DO.11.10 mice were infected with either γHV68.OVA or γHV68.LacZ for either 16 (A and C) or 42 (B) days. Peritoneal cells were harvested and subjected to limiting-dilution PCR assays. For panel C, DO.11.10 mice were treated with anti-CD4 or isotype control antibodies prior to infection with γHV68.OVA. Data were pooled from three independent experiments.

ifested by the capacity of these cells to delay virus-induced death and to effectively control viral replication. These findings demonstrate that helper function-independent antiviral effects of CD4 T cells may play a significant role in control of chronic herpesvirus infection. Consistent with this, we showed that preexisting immune CD4 T cells are capable of significantly decreasing the establishment of both early and late gammaherpesvirus latency. This finding contrasts with the ineffectiveness of CD8 T-cell responses in preventing the establishment of the late form of gammaherpesvirus latency (4, 37, 44).

The data presented here suggest that CD4 T cells may have independent antiviral function in the control of acute and latent γHV68 infection. However, the data presented here were derived from a system that used both a nonviral antigen and a high frequency of antiviral CD4 T cells. In addition, we chose to drive expression of OVA from the CMV immediate-early promoter so that the protein would be expressed at high levels, and we selected a form of OVA that is known to be efficiently presented to CD4 T cells (21). We designed this system to test what CD4 T cells could do under optimal conditions of high antigen expression and high CD4 T-cell frequency. Further experiments are needed to determine whether the mechanisms defined here extend to CD4 T cells specific for γHV68 antigens, such as the previously identified antigens ORF11 and gp150 (16). However, the relevance of these studies is substantiated by the demonstration that, in the absence of immune antibody, CD4 T cells play a critical role in vaccination against γHV68 latency (26a). Further experiments will also be necessary to determine whether the functions of CD4 T cells defined here are dependent on the route of inoculation, although establishment of latency is independent of both route and dose of inoculation (43).

Helper function-independent effects of CD4 T cells on γHV68 infection. The existence of B-cell- and CD8 T-cell-independent antiviral activity of CD4 T cells during γHV68 infection has been strongly suggested by studies using depletion of CD4 and CD8 T cells in B-cell-deficient mice (8). However, these B-cell-deficient mice might theoretically make physiologically important CD4-dependent antiviral immunoglobulin A responses (25). In addition, CD8 T cells may play a critical role in controlling γHV68 infection in these B-cell-deficient mice (8), and the role of CD8 T cells in CD4 T-cell function during γHV68 infection is controversial. For example, some studies have found no CD4 dependence of CD8 responses to γHV68 (3, 36). In contrast, other studies have argued for functional deficiencies in the CD8 T-cell response to γHV68 in the absence of CD4 T-cell help (7, 23). Thus, it might be that loss of CD4 T-cell help for the few CD8 T cells remaining in B-cell-deficient mice after anti-CD8 treatment might contribute to the effects of CD4 T cells in this system.

This being the case, we felt it important to determine, using a genetic approach, whether CD4 T cells can independently exhibit antiviral activity in the complete absence of CD8 T cells and B cells. We therefore developed a system utilizing infection with γHV68.OVA to take advantage of the availability of TCR tg mice containing CD4 T cells specific for OVA. Breeding these mice with RAG mice allowed analysis of CD4 T cells in the absence of CD8 T cells and B cells. It is possible that these TCR tg/RAG mice would have OVA-specific CD8 cells. However, we showed that the effects of tg expression in these mice required CD4 T cells and were not affected by administration of anti-CD8 antibody. Using these mice, we confirmed the conclusion of Christensen et al. (8) that CD4 T cells can exert B-cell- and CD8 T-cell-independent control of γHV68 infection.

Effects of CD4 T cells on latency. One of the most striking findings in the γHV68 system is that strong CD8 T-cell responses, even responses that dramatically reduced replication during acute infection, fail to prevent the establishment of normal levels of γHV68 latency (4, 37, 42). In contrast, vaccination with a reactivation-defective live attenuated mutant of γHV68 effectively diminishes (to below detectable levels) the establishment of both early and late γHV68 latency, even in CD8-deficient mice (42). This shows that there must be an immune mechanism or mechanisms that can prevent the establishment of latency but that immunization of CD8 T cells, at least for the antigens evaluated to date, is not effective in isolation to prevent establishment of the late form of γHV68 latency. Consistent with this, McClellan et al. report that depletion of CD4 T cells decreases the efficacy of vaccination against γHV68 latency (26a).

The potency of CD4 T cells in controlling acute γHV68 replication, even in the absence of B cells or CD8 T cells in the γHV68.OVA system, together with the fact that effective vaccination with a reactivation-deficient γHV68 mutant occurs in CD8-deficient mice (42), suggested to us that CD4 T cells specific for a virus-encoded antigen might, if present at high enough levels from the beginning of infection, inhibit the establishment of early and late γHV68 latency. While the TCR tg mice contain a higher frequency of antiviral CD4 T cells than seen after primary viral infection, vaccination would generate an increased number of immune CD4 T cells. We found that preexisting CD4 T cells specific for OVA can limit the establishment of both early and late γHV68 latency. Further experiments will be necessary to determine whether the CD4 T-cell-mediated reduction in latency observed in DO.11.10 mice requires B cells and/or CD8 T cells. We cannot determine whether a reduction of latently infected cells was seen in the absence of B cells and CD8 T cells (DO.11.10/RAG mice), as no latency was detected in γHV68.LacZ-infected mice due to the high level of productive infection. This also shows that it is not necessary to have preexisting responses to many different viral antigens or combined preexisting CD4, CD8, and B-cell responses to limit the establishment of latency.

Of interest is that the effectiveness of CD4 T cells in controlling γHV68 replication in DO11.10/RAG mice allowed us to detect latently infected cells in the absence of either CD8 T-cell responses or B-cell responses. This establishes that CD8 T cells and antibody responses are not required for the establishment of γHV68 latency in either macrophages or dendritic cells. These findings do not address the role of CD4 T cells in controlling latency in B cells, which are not present in RAG-deficient mice. However, it may be that inhibition of the establishment of latency, as observed in DO.11.10 mice, requires the presence of CD8 T cells or B cells present in these mice.

What CD4 T cells cannot do alone. While the studies that we present here show that high enough levels of virus-specific CD4 T cells can have significant effects on γHV68 replication, lethality, and latency, it is clear from our results that CD4 T cells specific for a single virus-encoded protein cannot completely control infection. For example, since CD4 T cells alone only delay lethal infection in DO.11.10/RAG mice, it is clear that cells other than CD4 T cells in DO.11.10 TCR tg mice must contribute to the prolonged survival of these mice (data not shown) compared to DO.11.10/RAG mice. The cause of death in DO.11.10/RAG mice is not known, and thus the reason that CD4 T cells alone are not sufficient to control infection remains to be determined. This issue is especially interesting since we observe very effective control of virus replication in spleen and liver by OVA-specific CD4 T cells as late as 16 days after infection, but the mice die about 10 days later. It is possible that viral mutants that evade CD4 T-cell control might arise in these mice. Alternatively, the quality and effectiveness of CD4 T-cell responses might wane in the absence of B cells or CD8 T cells.

Mechanism of CD4 T-cell action in the control of γHV68 infection. The data showing that CD4 T cells can control replication, prevent lethal infection, and inhibit the establishment of latency are consistent with other studies showing that CD4 T cells play a critical role in regulating chronic γHV68 infection (6, 7, 24, 51). Together with evidence that CD4 T cells can recognize Epstein-Barr virus latent antigens and both exhibit cytotoxicity and secrete IFN-γ, these data support the concept that CD4 T cells are critically important regulators of gammaherpesvirus latency (5, 28, 30, 31). This makes identification of mechanisms of CD4 T-cell action against gammaherpesviruses an important goal.

In this regard, the data presented here add helper-independent antiviral effects of CD4 T cells to standard helper functions as potential mechanisms for the effects of CD4 T cells on γHV68 pathogenesis. It will be important to define the mechanism(s) responsible for the B- and T-cell-independent effects on γHV68 infection observed here to understand their relative importance in CD4 T-cell function. Clearly, a lead candidate for a cytokine important for CD4 T-cell action is IFN-γ (8, 10, 51). Alternatively, CD4 T cells could act by directly lysing infected cells. For example, CD4 T cells isolated from Epstein-Barr virus-infected patients can lyse target cells in vitro (5, 28, 31).

A role for IFN-γ in CD4 T-cell function during latency is strongly suggested by studies using antibody to IFN-γ to demonstrate a critical role for this cytokine in control of γHV68 infection (8, 10). However, the cells responsible for IFN-γ secretion in these systems (e.g., CD4 T cells, CD8 T cells, or NK cells) have not been identified. It will be important to determine which cells secrete physiologically effective amounts of IFN-γ and to determine the IFN-γ dependence of helper and helper-independent functions of CD4 T cells to understand how IFN-γ secretion relates to CD4 T-cell function.

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REFERENCES

1. **Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone.** 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol. Cell Biol. **76:**34–40.
2. **Barnden, M. J., W. R. Heath, and F. R. Carbone.** 1997. Down-modulation of CD8 beta-chain in response to an altered peptide ligand enables developing thymocytes to escape negative selection. Cell. Immunol. **175:**111–119.
3. **Belz, G. T., H. Liu, S. Andreansky, P. C. Doherty, and P. G. Stevenson.** 2003. Absence of a functional defect in CD8+ T cells during primary murine gammaherpesvirus-68 infection of I-A(b-/-) mice. J. Gen. Virol. **84:**337–341.
4. **Belz, G. T., P. G. Stevenson, M. R. Castrucci, J. D. Altman, and P. C. Doherty.** 2000. Postexposure vaccination massively increases the prevalence of gamma-herpesvirus-specific CD8+ T cells but confers minimal survival advantage on CD4-deficient mice. Proc. Natl. Acad. Sci. USA **97:**2725–2730.
5. **Bickham, K., C. Munz, M. L. Tsang, M. Larsson, J. F. Fonteneau, N. Bhardwaj, and R. Steinman.** 2001. EBNA1-specific CD4+ T cells in healthy carriers of Epstein-Barr virus are primarily Th1 in function. J. Clin. Invest. **107:**121–130.
6. **Brooks, J. W., A. M. Hamilton-Easton, J. P. Christensen, R. D. Cardin, C. L. Hardy, and P. C. Doherty.** 1999. Requirement for CD40 ligand, CD4(+) T cells, and B cells in an infectious mononucleosis-like syndrome. J. Virol. **73:**9650–9654.
7. **Cardin, R. D., J. W. Brooks, S. R. Sarawar, and P. C. Doherty.** 1996. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. J. Exp. Med. **184:**863–871.
8. **Christensen, J. P., R. D. Cardin, K. C. Branum, and P. C. Doherty.** 1999. CD4(+) T cell-mediated control of a gamma-herpesvirus in B cell-deficient mice is mediated by IFN-gamma. Proc. Natl. Acad. Sci. USA **96:**5135–5140.
9. **Cobbold, S. P., A. Jayasuriya, A. Nash, T. D. Prospero, and H. Waldmann.** 1984. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. Nature **312:**548–551.
10. **Dal Canto, A. J., P. E. Swanson, A. K. O'Guin, S. H. Speck, and H. W. Virgin.** 2001. IFN-gamma action in the media of the great elastic arteries, a novel immunoprivileged site. J. Clin. Investig. **107:**R15–R22.

11. **Deshpande, S., M. Zheng, S. Lee, K. Banerjee, S. Gangappa, U. Kumaraguru, and B. T. Rouse.** 2001. Bystander activation involving T lymphocytes in herpetic stromal keratitis. J. Immunol. **167:**2902–2910.
12. **Ehtisham, S., N. P. Sunil-Chandra, and A. A. Nash.** 1993. Pathogenesis of murine gammaherpesvirus infection in mice deficient in CD4 and CD8 T cells. J. Virol. **67:**5247–5252.
13. **Flaño, E., S. M. Husain, J. T. Sample, D. L. Woodland, and M. A. Blackman.** 2000. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. J. Immunol. **165:**1074–1081.
14. **Flaño, E., I. J. Kim, J. Moore, D. L. Woodland, and M. A. Blackman.** 2003. Differential gamma-herpesvirus distribution in distinct anatomical locations and cell subsets during persistent infection in mice. J. Immunol. **170:**3828–3834.
15. **Flaño, E., D. L. Woodland, and M. A. Blackman.** 1999. Requirement for CD4+ T cells in V beta 4+CD8+ T cell activation associated with latent murine gammaherpesvirus infection. J. Immunol. **163:**3403–3408.
16. **Flaño, E., D. L. Woodland, M. A. Blackman, and P. C. Doherty.** 2001. Analysis of virus-specific $CD4^+$ T cells during long-term gammaherpesvirus infection. J. Virol. **75:**7744–7748.
17. **Gangappa, S., S. B. Kapadia, S. H. Speck, and H. W. Virgin IV.** 2002. Antibody to a lytic cycle viral protein decreases gammaherpesvirus latency in B-cell-deficient mice. J. Virol. **76:**11460–11468.
18. **Gangappa, S., L. F. Van Dyk, T. J. Jewett, S. H. Speck, and H. W. Virgin IV.** 2002. Identification of the in vivo role of a viral bcl-2. J. Exp. Med. **195:**931–940.
19. **Kim, I. J., E. Flaño, D. L. Woodland, and M. A. Blackman.** 2002. Antibody-mediated control of persistent γ-herpesvirus infection. J. Immunol. **168:** 3958–3964.
20. **Kim, I. J., E. Flaño, D. L. Woodland, F. E. Lund, T. D. Randall, and M. A. Blackman.** 2003. Maintenance of long term gamma-herpesvirus B cell latency is dependent on CD40-mediated development of memory B cells. J. Immunol. **171:**886–892.
21. **Kurts, C., F. R. Carbone, M. Barnden, E. Blanas, J. Allison, W. R. Heath, and J. F. Miller.** 1997. CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. J. Exp. Med. **186:**2057–2062.
22. **Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka.** 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. J. Exp. Med. **184:**923–930.
23. **Liu, H., S. Andreansky, G. Diaz, T. Hogg, and P. C. Doherty.** 2002. Reduced functional capacity of CD8+ T cells expanded by post-exposure vaccination of gamma-herpesvirus-infected CD4-deficient mice. J. Immunol. **168:**3477–3483.
24. **Liu, L., E. J. Usherwood, M. A. Blackman, and D. L. Woodland.** 1999. T-cell vaccination alters the course of murine herpesvirus 68 infection and the establishment of viral latency in mice. J. Virol. **73:**9849–9857.
25. **Macpherson, A. J., A. Lamarre, K. McCoy, G. R. Harriman, B. Odermatt, G. Dougan, H. Hengartner, and R. M. Zinkernagel.** 2001. IgA production without mu or delta chain expression in developing B cells. Nat. Immunol. **2:**625–631.
26. **Marques, S., S. Efstathiou, K. G. Smith, M. Haury, and J. P. Simas.** 2003. Selective gene expression of latent murine gammaherpesvirus 68 in B lymphocytes. J. Virol. **77:**7308–7318.
26a. **McClellan, J. S., S. A. Tibbetts, S. Gangappa, K. A. Brett, and H. W. Virgin IV.** 2004. Critical role of CD4 T cells in an antibody-independent mechanism of vaccination against gammaherpesvirus latency. J Virol. **78:**6836–6845.
27. **Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou.** 1992. RAG-1-deficient mice have no mature B and T lymphocytes. Cell **68:**869–877.
28. **Munz, C., K. L. Bickham, M. Subklewe, M. L. Tsang, A. Chahroudi, M. G. Kurilla, D. Zhang, M. O'Donnell, and R. M. Steinman.** 2000. Human CD4(+) T lymphocytes consistently respond to the latent Epstein-Barr virus nuclear antigen EBNA1. J. Exp. Med. **191:**1649–1660.
29. **Murphy, K. M., A. B. Heimberger, and D. Y. Loh.** 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. Science **250:**1720–1723.
30. **Omiya, R., C. Buteau, H. Kobayashi, C. V. Paya, and E. Celis.** 2002. Inhibition of EBV-induced lymphoproliferation by CD4(+) T cells specific for an MHC class II promiscuous epitope. J. Immunol. **169:**2172–2179.
31. **Paludan, C., K. Bickham, S. Nikiforow, M. L. Tsang, K. Goodman, W. A. Hanekom, J. F. Fonteneau, S. Stevanovic, and C. Munz.** 2002. Epstein-Barr nuclear antigen 1-specific CD4(+) Th1 cells kill Burkitt's lymphoma cells. J. Immunol. **169:**1593–1603.
32. **Radka, S. F., C. Machamer, P. Cresswell, D. D. Kostyu, F. E. Ward, and D. B. Amos.** 1983. SFR3-DR5, a monoclonal antibody with HLA-DR5 specificity. J. Immunol. **130:**1863–1866.
33. **Robertson, J. M., P. E. Jensen, and B. D. Evavold.** 2000. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323–339 epitope. J. Immunol. **164:**4706–4712.
34. **Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, and A. M. Stall.** 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J. rearrangement. Cell **68:**855–867.
35. **Smith, S. C., and P. M. Allen.** 1991. Myosin-induced acute myocarditis is a T cell-mediated disease. J. Immunol. **147:**2141–2147.
36. **Stevenson, P. G., G. T. Belz, J. D. Altman, and P. C. Doherty.** 1998. Virus-specific CD8(+) T cell numbers are maintained during gamma-herpesvirus reactivation in CD4-deficient mice. Proc. Natl. Acad. Sci. USA **95:**15565–15570.
37. **Stevenson, P. G., G. T. Belz, M. R. Castrucci, J. D. Altman, and P. C. Doherty.** 1999. A gamma-herpesvirus sneaks through a CD8(+) T cell response primed to a lytic-phase epitope. Proc. Natl. Acad. Sci. USA **96:**9281–9286.
38. **Stevenson, P. G., and P. C. Doherty.** 1999. Non-antigen-specific B-cell activation following murine gammaherpesvirus infection is CD4 independent in vitro but CD4 dependent in vivo. J. Virol. **73:**1075–1079.
39. **Stewart, J. P., E. J. Usherwood, A. Ross, H. Dyson, and T. Nash.** 1998. Lung epithelial cells are a major site of murine gammaherpesvirus persistence. J. Exp. Med. **187:**1941–1951.
40. **Sunil-Chandra, N. P., J. Arno, J. Fazakerley, and A. A. Nash.** 1994. Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. Am. J. Pathol. **145:**818–826.
41. **Tibbetts, S. A., J. Loh, V. van Berkel, J. S. McClellan, M. A. Jacoby, S. B. Kapadia, S. H. Speck, and H. W. Virgin IV.** 2003. Establishment and maintenance of gammaherpesvirus latency are independent of infective dose and route of infection. J. Virol. **77:**7696–7701.
42. **Tibbetts, S. A., J. S. McClellan, S. Gangappa, S. H. Speck, and H. W. Virgin IV.** 2003. Effective vaccination against long-term gammaherpesvirus latency. J. Virol. **77:**2522–2529.
43. **Tibbetts, S. A., L. Van Dyk, S. H. Speck, and H. W. Virgin IV.** 2002. Immune control of the number and reactivation phenotype of cells latently infected with a gamma-herpesvirus. J. Virol. **76:**7125–7132.
44. **Usherwood, E. J., D. J. Roy, K. Ward, S. L. Surman, B. M. Dutia, M. A. Blackman, J. P. Stewart, and D. L. Woodland.** 2000. Control of gammaherpesvirus latency by latent antigen-specific CD8(+) T cells. J. Exp. Med. **192:**943–952.
45. **Usherwood, E. J., K. A. Ward, M. A. Blackman, J. P. Stewart, and D. L. Woodland.** 2001. Latent antigen vaccination in a model gammaherpesvirus infection. J. Virol. **75:**8283–8288.
46. **Van Dyk, L. F., J. L. Hess, J. D. Katz, M. Jacoby, S. H. Speck, and H. W. Virgin IV.** 1999. The murine gammaherpesvirus 68 v-cyclin is an oncogene that promotes cell cycle progression in primary lymphocytes. J. Virol. **73:** 5110–5122.
47. **Van Dyk, L. F., H. W. Virgin IV, and S. H. Speck.** 2000. The murine gammaherpesvirus 68 v-cyclin is a critical regulator of reactivation from latency. J. Virol. **74:**7451–7461.
48. **Van Dyk, L. F., H. W. Virgin IV, and S. H. Speck.** 2003. Maintenance of gammaherpesvirus latency requires viral cyclin in the absence of B lymphocytes. J. Virol. **77:**5118–5126.
49. **Verma, S. C., and E. S. Robertson.** 2003. Molecular biology and pathogenesis of Kaposi sarcoma-associated herpesvirus. FEMS Microbiol. Lett. **222:**155–163.
50. **Weck, K. E., M. L. Barkon, L. I. Yoo, S. H. Speck, and H. W. Virgin IV.** 1996. Mature B cells are required for acute splenic infection, but not for establishment of latency, by murine gammaherpesvirus 68. J. Virol. **70:**6775–6780.
51. **Weck, K. E., A. J. Dal Canto, J. D. Gould, A. K. O'Guin, K. A. Roth, J. E. Saffitz, S. H. Speck, and H. W. Virgin.** 1997. Murine gammaherpesvirus 68 causes severe large vessel arteritis in mice lacking interferon-gamma responsiveness: a new model for virus induced vascular disease. Nat. Med. **3:**1346–1353.
52. **Weck, K. E., S. S. Kim, H. W. Virgin IV, and S. H. Speck.** 1999. B cells regulate murine gammaherpesvirus 68 latency. J. Virol. **73:**4651–4661.
53. **Weck, K. E., S. S. Kim, H. W. Virgin IV, and S. H. Speck.** 1999. Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. J. Virol. **73:**3273–3283.
54. **Willer, D. O., and S. H. Speck.** 2003. Long-term latent murine gammaherpesvirus 68 infection is preferentially found within the surface immunoglobulin D-negative subset of splenic B cells in vivo. J. Virol. **77:**8310–8321.
55. **Young, L. S., and P. G. Murray.** 2003. Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene **22:**5108–5121.